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1 TITLE PAGE

Title: Analysis of Scotch Whisky by ^1H NMR and chemometrics yields insight into its complex chemistry

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2 ABSTRACT

Scotch Whisky has been analysed as a complex mixture in its raw form using high resolution Nuclear Magnetic Resonance (NMR) and previously developed water and ethanol suppression techniques. This has allowed for the positive identification of 25 compounds in Scotch Whisky by means of comparison to reference standards, spike-in experiments, and advanced 1D and 2D NMR experiments. Quantification of compounds was hindered by signal overlap, though peak alignment strategies were largely successful. Statistical total correlation spectroscopy (STOCSY) yielded information on signals arising from the same compound or compounds of similar origin. Statistical analysis of the spectra was performed using Independent and Principal Components Analysis (ICA, PCA) as well as Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA). Several whisky production parameters were successfully modelled, including blend or malt status, use of peated malt, alcohol strength, generic authentication and maturation wood type, whilst age and geographical origin could not be modelled.

2.1 HIGHLIGHTS

- NMR can inform the chemistry of Scotch Whisky production
- Twenty-five compounds were identified by NMR, some in different forms
- Statistical analysis successfully classified several whisky parameters
- NMR can be used for generic Scotch Whisky authentication

2.2 HYPOTHESES

^1H NMR spectra of Scotch Whisky reflect many aspects of its chemistry and production.

2.3 CHEMICAL COMPOUNDS STUDIED IN THIS ARTICLE:

Ethanol (PubChem CID: 702), glucose (PubChem CID: 5793), fructose (PubChem CID: 5984), 2-phenylethanol (PubChem CID: 6054), syringaldehyde (PubChem CID: 8655), syringic acid (PubChem

CID: 10742), gallic acid (PubChem CID: 370), 5-HMF (PubChem CID: 237332), furfural (PubChem CID: 7362), ethyl formate (PubChem CID: 8025), formic acid (PubChem CID: 284), ethyl acetate (PubChem CID: 8857), acetaldehyde (PubChem CID: 177), 3-methylbutanol (PubChem CID: 31260), 2-methylbutanol (PubChem CID: 8723), isobutanol (PubChem CID: 6560), n-butanol (PubChem CID: 263), n-propanol (PubChem CID: 1031)

2.4 KEYWORDS

Scotch Whisky, NMR, Complex Mixtures, Chemometrics, Beverages

2.5 LIST OF ABBREVIATIONS

COSY – Correlation Spectroscopy
CV-ANOVA – Cross Validated Analysis of Variance
DOSY – Diffusion Ordered Spectroscopy
ESI – Electrospray Ionisation
FID – Flame Ionisation Detector
FTICR-MS – Fourier transform Ion Cyclotron Resonance Mass Spectrometry
GC – Gas Chromatography
HSQC – Heteronuclear Single Quantum Coherence
LC – Liquid Chromatography
MS – Mass Spectrometry
NOESY – Nuclear Overhauser Effect Spectroscopy
NMR – Nuclear Magnetic Resonance
ICA – Independent Components Analysis
OPLS-DA – Orthogonal Partial Least Squares Discriminant Analysis
PCA – Principal Components Analysis
REST – Relaxation Encoded Spectroscopy
STOCSY – Statistical Total Correlation Spectroscopy
SWRI – Scotch Whisky Research Institute
TOCSY – Total Correlation Spectroscopy

1 INTRODUCTION

Chemically, Scotch Whisky is a complex mixture comprising thousands of compounds (Kew, Goodall, Clarke, & Uhrin, 2017). As a product, Scotch Whisky has a significant cultural and economic value. Production of Scotch Whisky involves several key stages (R. Aylott & Mackenzie, 2010). Briefly, it is produced from the fermentation, distillation, and maturation of cereal sources – malted barley in the case of malt whisky, and other cereals for grain whisky. The final product may originate from a single distillery (single malt or single grain) or be the product of multiple distilleries (blended malt or blended grain). Blends of malt and grains are called blended Scotch Whisky. As per the Scotch Whisky Regulations 2009 (UK Statutory Instrument No. 2890), Scotch must be matured in oak barrels in Scotland for a minimum of 3 years, and, where an age statement is listed, this corresponds to the youngest spirit in the bottle. Most commonly, Scotch is matured in barrels previously used for the maturation of Bourbon whiskey or production of Sherry wines. Other cask types can be used, including ale, port, or other wines. Some whiskies are initially matured in one cask, and then ‘finished’ in a secondary cask for a shorter time (Piggot, Conner, Paterson, & Clyne, 1993). Scotch Whisky is bottled at a minimum 40% alcohol-by-volume (ABV).

Research into the chemistry of Scotch Whisky has previously been conducted using more traditional techniques such as gas-chromatography (GC) or liquid chromatography (LC) coupled to various detectors, including flame ionisation detection (FID), UV-Vis, mass spectrometry (MS), or even olfactory (O) in the case of GC for sensory analysis (R. I. Aylott, Clyne, Fox, & Walker, 1994; R. Aylott & Mackenzie, 2010; MacKenzie & Aylott, 2004). The reader is referred to a 2015 review of the chemical analysis of whisky (Wiśniewska, Dymerski, Wardencki, & Namieśnik, 2015). These methods are highly targeted, and, whilst they are refined, often highly reproducible, and quantitative, they suffer from potentially long run times, substantial time and effort in method development, and the limited range of compounds which are accessible via individual methods.

Untargeted MS approaches have been utilised for whisky analysis, including high-resolution Fourier transform Ion Cyclotron Resonance (FTICR) MS. In a previous paper (Kew, Goodall, et al., 2017), we examined the chemistry and chemical diversity of 85 Scotch Whisky samples by electrospray (ESI) FTICR-MS, and identified thousands of molecular formulas, including potential markers for production styles. Similarly, this approach has been used by other groups studying whisky (Garcia et al., 2013; Roullier-Gall et al., 2018). However, this technique is not quantitative, nor does it provide any structural information in its routine use.

The analysis of alcoholic beverages by NMR has been performed before. Monakhova *et al.* implemented a line-selective solvent suppression technique for the acquisition of NMR spectra of alcoholic samples, such as wine (Monakhova et al., 2011). Other alcoholic spirits have been investigated by NMR, including Greek grape marc spirits (Fotakis et al., 2013); their fingerprinting allowed for authentication by chemometric techniques (Fotakis & Zervou, 2016). Targeted analysis of alcoholic drinks has also been performed by NMR, for example for the detection of ethyl carbamate in spirits (Monakhova, Kuballa, & Lachenmeier, 2012). The fate of acetaldehyde has been investigated in wine using NMR and GC-MS (Peterson, 2017). NMR and diffusion ordered spectroscopy (DOSY) has been used for the analysis of Port wine (Nilsson et al., 2004), whilst relaxation encoded NMR (REST) has been used for the analysis of beer (Dal Poggetto, Castañar, Adams, Morris, & Nilsson, 2017). Beyond alcoholic beverages, similar approaches to analyses of mixtures of small molecules by NMR have been reported for other food products, including honey (Spiteri et al., 2015) and fruit juices (Belton et al., 1997; Spraul et al., 2009)..

One major driver of research into Scotch Whisky is authentication and product protection. Authentication studies can be divided into two broad types – generic and brand. Generic authentication aims to ensure that a product claiming to be Scotch Whisky is consistent with this spirit drink category, whilst brand authentication aims to tell if a product is consistent with a specific Scotch

Whisky brand. Recent work utilised NMR spectroscopy for brand authentication of limited sample sets of Scotch Whisky, vodka, and rum acquired from Russia, Kenya, and Germany (Kuballa et al., 2018).

Many of these studies utilise chemometric, or statistical, techniques to leverage more information from the acquired data. The most common method is Principal Components Analysis (PCA), an unsupervised data dimensionality reduction and visualisation technique. PCA identifies new variables (principal components, linear combinations of input variables) which describe the maximum variation of the input data. Supervised techniques, such as Partial Least Squares Discriminant Analysis (PLS-DA) or orthogonal PLS-DA (OPLS-DA), are commonly used for classification of samples. PLS-DA is reviewed and discussed in two recent papers, including a discussion of potential shortcomings of this method (Brereton & Lloyd, 2014; Gromski et al., 2015). OPLS-DA is a powerful methodology that separates out significant 'orthogonal' variation to improve the classification ability of the generated model. A recent review discusses trends in chemometrics in food authentication (Granato et al., 2018). Independent component analysis (ICA) has been used for fingerprinting wine (Monakhova, Godelmann, Kuballa, Mushtakova, & Rutledge, 2015). ICA is a blind source separation technique and can be used to identify individual components within a mixed signal. . Statistical Total Correlation Spectroscopy (STOCSY) allows for multiple similar spectra to be used to determine correlations between signals (Cloarec et al., 2005).. STOCSY exploits the quantitative nature of NMR; it identifies signals from the same, or separate compounds, that increase or decrease proportionally to their concentration. . It will also inform about molecules with the same origin pathway.

Recently, we developed an advanced "solvent" (water and ethanol) suppression methodology to allow for the automated acquisition of NMR spectra of Scotch Whisky (Kew, Bell, Goodall, & Uhrín, 2017). This method, built on the commonly used NOESY-presaturation experiment (Mckay, 2011), selectively suppresses the water and ethanol proton signals, including the ^{13}C satellites of ethanol. This allows for the acquisition of NMR spectra of whisky, with limited sample preparation, revealing dozens of congeners. Our approximate limit of detection in that study was $50\text{ }\mu\text{M}$,. Implementation of the solvent

suppression technique into several other 1D and 2D NMR experiments allowed for the development of a toolkit for the identification of compounds in whisky and other high-alcohol strength products.

Here we present the NMR assignment of 25 compounds within the matrix of Scotch Whisky and discuss the quantification of compounds in whisky by NMR. We use chemometric methods, PCA and OPLS-DA, to investigate if the ^1H NMR spectra of Scotch Whisky reflect the provenance of whisky, including the class (blend or malt) and maturation wood type (Bourbon or Sherry), and allow for generic authentication.

2 MATERIALS AND METHODS

Scotch Whisky samples ($n=148$) were provided by the Scotch Whisky Research Institute (SWRI). Every two years the SWRI requests around 50 Scotch Whisky brands from its member companies. These brands are selected by the SWRI to represent the breadth and volume of Scotch Whisky in production and are sourced directly from the producers to guarantee provenance. The Scotch Whisky samples in this work were subsampled (15 ml in sealed vials) at the SWRI, from the carefully maintained sample sets collected in 2010, 2012 and 2014, and transferred to University of Edinburgh. These sampled brands represented (r , %) of Scotch Whisky UK and export case sales (2010, $n=47$, $r=67$ %; 2012, $n=49$, $r=71$ %; and 2014, $n=52$, $r=73$ %). For a complete list of anonymised samples see Table S1 in Supplementary Material. Known counterfeit samples ($n=32$) were also provided by the SWRI. Their origin and counterfeit status determination is discussed in the Supplementary Material. DSS- d_6 (98 %), D_2O (99 %), acetic acid- d_4 (99.5 %), and sodium acetate- d_3 (99 %) were acquired from Sigma-Aldrich Co.

2.1 NMR ANALYSES

Samples were prepared, and experiments performed, as previously described (Kew, Bell, et al., 2017). Briefly, whisky (500 μL) was mixed with an acetic acid/sodium acetate buffer in D_2O (100 μL) containing

DSS, yielding final concentrations of 25 mM buffer and 1 mM DSS-d₆ in a 5 mm NMR tube. NMR experiments were performed at 600 MHz on a Bruker Avance III spectrometer equipped with a cryogenically cooled TCI probe, 16-position sample changer and automatic tuning and matching.

For 1D ¹H analysis, a modified NOESY-presaturation sequence was used with selective saturation of ethanol CH₂ and CH₃ signals on the first channel, selective ethanol carbon decoupling on the second, and water OH saturation on the third. For other NMR experiments, a similar presaturation block was prepended to standard Bruker NMR pulse sequences. For further detail, refer to our previous paper (Kew, Bell, et al., 2017). A summary of experimental parameters is presented in the Supplementary material.

NMR data were processed in Bruker TopSpin 3.5, including phasing and baseline correction. In the 1D ¹H NMR spectra, peaks were aligned using the *icoshift* algorithm (Savorani, Tomasi, & Engelsen, 2010). Compounds were identified by several methods including comparison to reference standards (provided by SWRI), spike-in experiments, and additional 1D and 2D NMR experiments.

2.2 STATISTICAL ANALYSES

1D ¹H NMR spectra were exported to a binned data matrix using MestreNova 12, with a bin size of 0.001 ppm. Regions containing residual suppressed solvent were removed, along with noise at either end of the spectrum, yielding a final matrix **X** (*m* x *n*) of *m* variables (9673) and *n* observations (148). Using this data matrix, a median NMR spectrum was calculated for visualisation of statistical models using Python to linearly combine and average the data. No data normalisation, unless otherwise mentioned, was performed as samples were prepared and run with identical experimental parameters.

STOCSY and ICA analyses were performed with Python (3.6). Here, STOCSY calculates the correlation coefficient between a defined ‘driver’ peak and all other signals in the spectrum. This was calculated

using the '*corrwith*' function of the Pandas (0.23.4) package for Python. ICA was calculated using the '*FastICA*' algorithm of the scikit-learn (0.19.1) package for Python.

Further statistical analyses (PCA, OPLS-DA) was performed in SIMCA 14.1 (Sartorius Stedim Biotech). PCA and OPLS-DA models were constructed with Pareto scaling. R^2 values quantify how much of the original data is described by the model, whilst Q^2 values represent the goodness of the model as calculated by a cross-fold validation. OPLS-DA models were further validated by permutation testing and CV-ANOVA, yielding *p*-values indicative of the model's ability to correctly classify samples. Except where noted, for all models all the spectral data was used.

3 RESULTS

3.1 NMR COMPOUND IDENTIFICATION

All ^1H NMR spectra of Scotch Whisky share common features across the chemical shift range from approximately 0.8 ppm through to 10 ppm. These include higher alcohols – such as 2- and 3-methyl butanol, isobutanol, n-propanol – carbohydrates – including glucose and fructose – and aromatic cask extractives, such as syringaldehyde and gallic acid. A total of 25 chemical structures were positively identified in Scotch Whisky, summarised in Table S2. A representative NMR spectrum of Scotch Whisky is presented in Figure 1, with annotations of major signals detailed in Table 1. For simplicity, not all identified signals are labelled here. The complete set of identified NMR data is tabulated in Table S2. As the carbohydrate region of the 1D spectrum is complex, the annotated HSQC spectrum for the carbohydrate region is included in Figure S1 and Table S3. Reference 1D ^1H spectra of higher alcohols are included in Figure S2, whilst an example of the spike-in method of compound identification is provided in Figure S3.

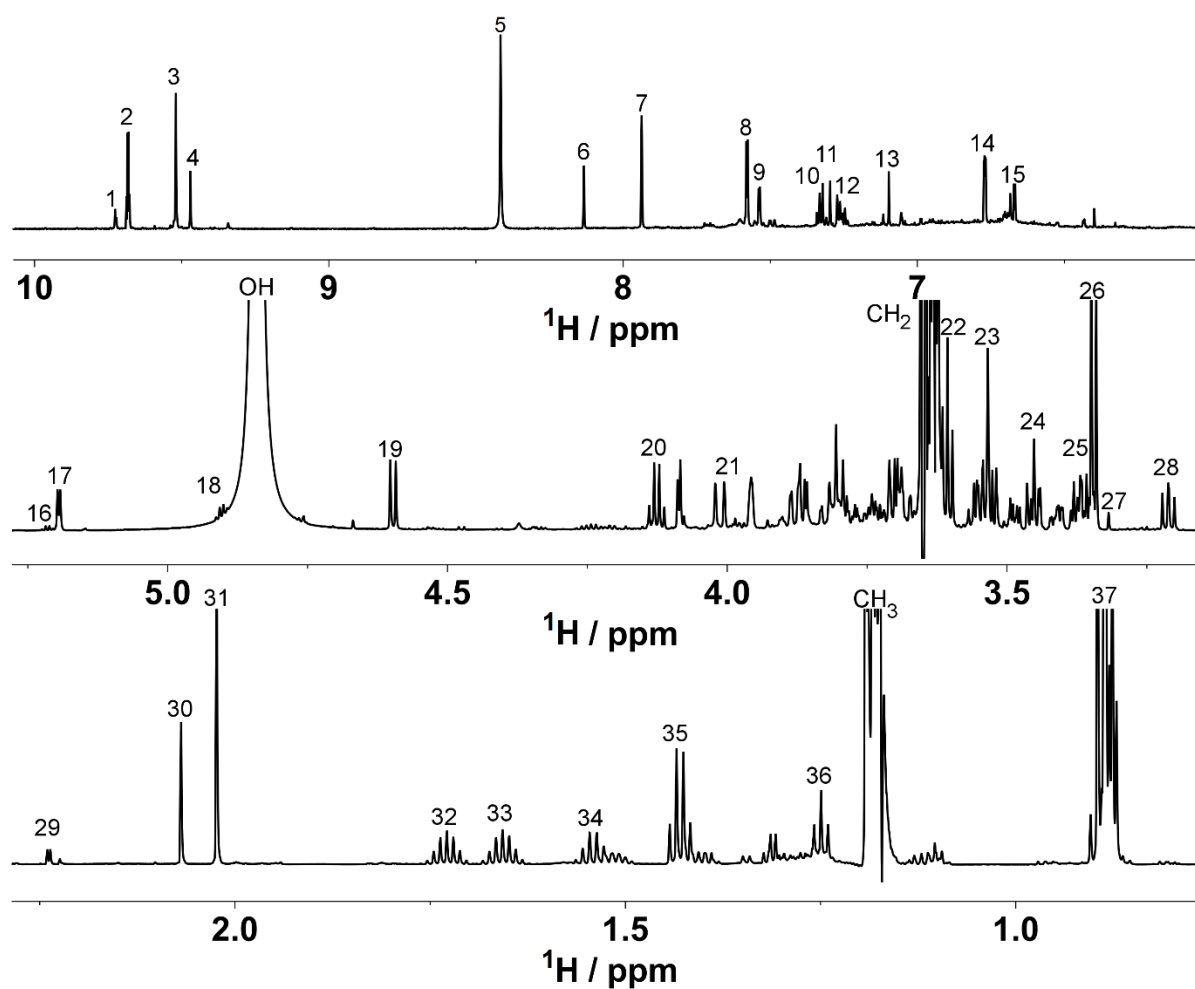


Figure 1 – Annotated 1D ^1H NMR spectrum of a Scotch Whisky sample (S10-1313). For simplicity, only major signals have been annotated, see the SI for full assignments, and Table 1 for annotation details.

#	ID	#	ID
1	Syringaldehyde	20	Ethyl acetate
2	Acetaldehyde	21	β -D-fructopyranose
3	Furfural	22	3-Methylbutanol
4	5-HMF	23	n-Propanol
5	Formic acid	24	2-Methylbutanol
6	Ethyl formate	25	2-Methylbutanol
7	Furfural	26	Iso-butanol
8	Furfural	27	Methanol
9	5-HMF	28	Glucose
10	2-Phenylethanol	29	Acetaldehyde
11	Syringaldehyde	30	Ethyl acetate
12	2-Phenylethanol	31	Acetate/acetic acid
13	Gallic acid	32	Iso-butanol
14	Furfural	33	3-Methylbutanol
15	5-HMF	34	n-Propanol
16	Acetaldehyde ethyl hemiacetal	35	3-Methylbutanol
17	α -D-glucopyranose	36	Ethyl acetate
18	Acetaldehyde water hemiacetal	37	Higher alcohol methyls
19	β -D-glucopyranose		

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208 Several compounds in Scotch Whisky exist in multiple forms. These include monosaccharides glucose

209 and fructose, which exist as α - or β - pyranose or furanose. The relative ratio of individual anomeric

210 configurations appears to be pH and ethanol strength dependent, hindering quantification of

compounds. For example, in a purely aqueous solution the ratio of α : β -D-glucopyranose is known to be 1:2.1 (Zhu, Zajicek, & Serianni, 2001), whilst in Scotch Whisky (minimum 40% ethanol v/v) we measured the ratios ranging from 1:2 to 1:1, based on integration of the signals of anomeric protons. Note that overlapping signals and proximity to the suppressed OH signal may impede the accuracy of these values.

Acetaldehyde is known to be an important compound in the maturation pathway of Scotch Whisky, formed by the oxidation of ethanol. However, in alcoholic solutions, such as whisky, acetaldehyde will form hemiacetals with both water and ethanol. The equilibrium constants are not known and not easily measured as the signals from the hemiacetals overlap with the suppressed signals of ethanol and water. Identities of these hemiacetals (acetaldehyde hydrate and acetaldehyde ethyl hemiacetal) were confirmed using model solutions, 1D ^{13}C NMR, and exchange suppressing DOSY NMR sequences (Aguilar, Adams, Nilsson, & Morris, 2014); spectra shown in Figure S4. Interestingly, the acetaldehyde diethyl acetal (1,1-diethoxyethane), known from other techniques to exist in Scotch Whisky (Lee, Paterson, Piggott, & Richardson, 2001), was not positively identified in the NMR spectra of whisky. It is probable that other alcohols and aldehydes within Scotch Whisky also form hemiacetals, although at such low levels that they are not detectable by NMR.

The multiple-form problem hinders quantification of the minor compounds in Scotch Whisky via NMR, as does signal overlap. Various software packages were evaluated to automate the accurate quantification of minor compounds in Scotch Whisky, however none performed sufficiently well to report in this work. This is an ongoing area of research.

3.2 STATISTICAL ANALYSIS OF NMR DATA

In the absence of quantitative data for NMR detectable compounds in Scotch Whisky, statistical analysis was performed on the raw spectra. To reduce variation of pH-dependent chemical shifts, each sample was buffered. Residual chemical shift variations – due to ethanol strength or concentration

effects – were compensated for in post-processing by means of peak alignment using *icoshift* (Savorani et al., 2010). This algorithm, written for Matlab, was implemented into an interactive tool for TopSpin using GNU Octave as a backend. Effects of peak alignment are shown in Figure S5. NMR spectra are routinely binned prior to statistical analysis to compensate for any residual chemical shift variation, and to reduce the data size for computation. With the successful use of the peak alignment algorithm, and modern computer hardware, a very fine level of binning (0.001 ppm) was achievable. This yielded a data matrix at an optimal compromise of size and resolution, retaining all the resolvable coupling information, as illustrated in Figure S6.

3.2.1 STOCSY can inform the chemistry of Scotch Whisky production

STOCSY can be used to determine correlations between signals from the same compound and from compounds of the same origin, i.e. co-products of fermentation or maturation. 1D STOCSY NMR spectra were produced by calculating the square of the Pearson correlation coefficient between a specific driver peak and all other signals in the spectrum, and colour coded into the median Scotch Whisky NMR spectrum.

An example is shown in

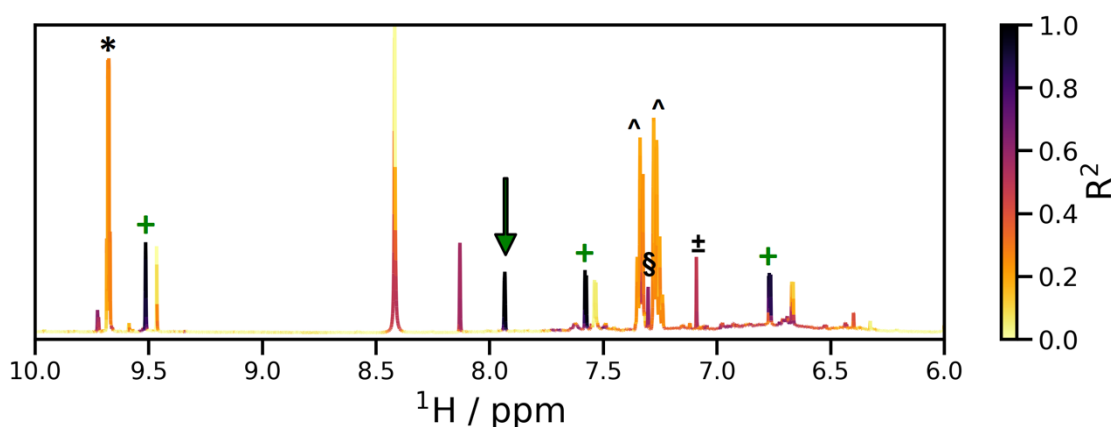


Figure 2 using the congener furfural as a driver compound (specifically one of its aromatic protons at 7.93 ppm). Furfural is largely maturation related, but some is produced during distillation. The figure shows R^2 values close to or equal to 1 for other protons of furfural (indicated with green crosses) – as

expected – and moderate correlations to some other signals, for example minor signals at 9.72 and 7.30 ppm which correspond to the cask extractive syringaldehyde, gallic acid at 7.1 ppm, and to many of the low-level unresolved signals between 6.5 and 7.7 ppm. Those signals must belong to other maturation compounds, whilst acetaldehyde (9.68 ppm) has a weaker correlation due to its formation during fermentation as well as maturation. The fermentation origins also explain the weaker correlations to signals such as 2-phenylethanol (7.33 and 7.26 ppm). Ethyl formate (8.1 ppm) is weakly correlated, as is formic acid (8.4 ppm) – the weaker correlation of formic acid will be due to its broader and more variable line shape in the spectra, thus reducing the correlation coefficient.

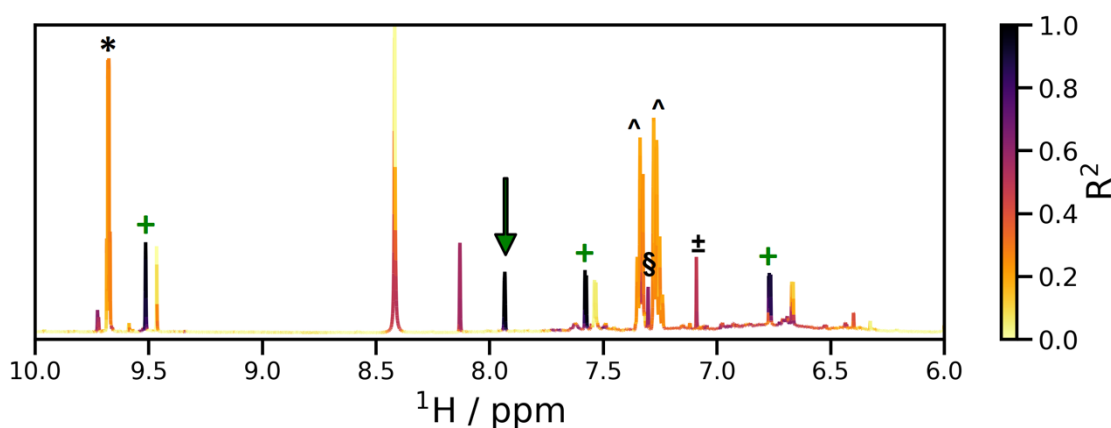


Figure 2 – 1D STOCSY NMR spectrum using furfural (7.93 ppm, arrow) as a driver peak. Correlation coefficients to the other signals in the median Scotch Whisky NMR spectrum are colour encoded. Other signals belonging to furfural are indicated with + symbols, acetaldehyde is shown with *, 2-phenylethanol with ^ symbol, syringaldehyde with § symbol and gallic acid with ± symbol.

A similar approach using 3-methyl butanol as a driver is reported in Figure S7. There, 3-methyl butanol correlates well to iso-butanol, another fermentation by-product. However, it has an R^2 close to 0 with n-propanol. This is because the sample set contains a mix of single and blended malt whiskies, and blended whisky. The latter contains high levels of grain spirit, which is known to contain lower levels of 2- and 3-methyl butanol, slightly higher levels of n-propanol, and similar levels of iso-butanol to

malt spirit (R. Aylott & Mackenzie, 2010). Therefore, correlations between the methylbutanols and n-propanol will be affected by the mixed sample set.

STOCSY analysis therefore allows to better understand the origins and relationships of compounds in Scotch Whisky using just 1D ^1H NMR spectra of final products, as well as has a potential to determine the identities of as-yet unidentified species.

Independent component analysis (ICA) has previously been used for the analysis of NMR spectra of mixtures (Monakhova et al., 2015). It can perform so-called 'blind source separation', and theoretically resolve the signals of n independent components in a mixture, provided there are $n+1$ measurements of the mixture signal. Thus, in this example, up to 147 independent components (or compound spectra) could theoretically be deconvolved. In practice, ICA is imperfect, and the output depends significantly on the input parameters. That said, in the example shown in Figure S8, the pure component spectra of three individual compounds could be isolated from the original whisky spectra.

3.2.2 PCA discriminates blended and malt whiskies

PCA is an unsupervised technique, however colour coding of the samples in a scores plot can allow easier visualisation of any underlying structure in the data. In Figure 3, two-dimensional scores plots are shown for the constructed PCA model for components 1 and 2 (Figure 3a) and 1 and 3 (Figure 3b).

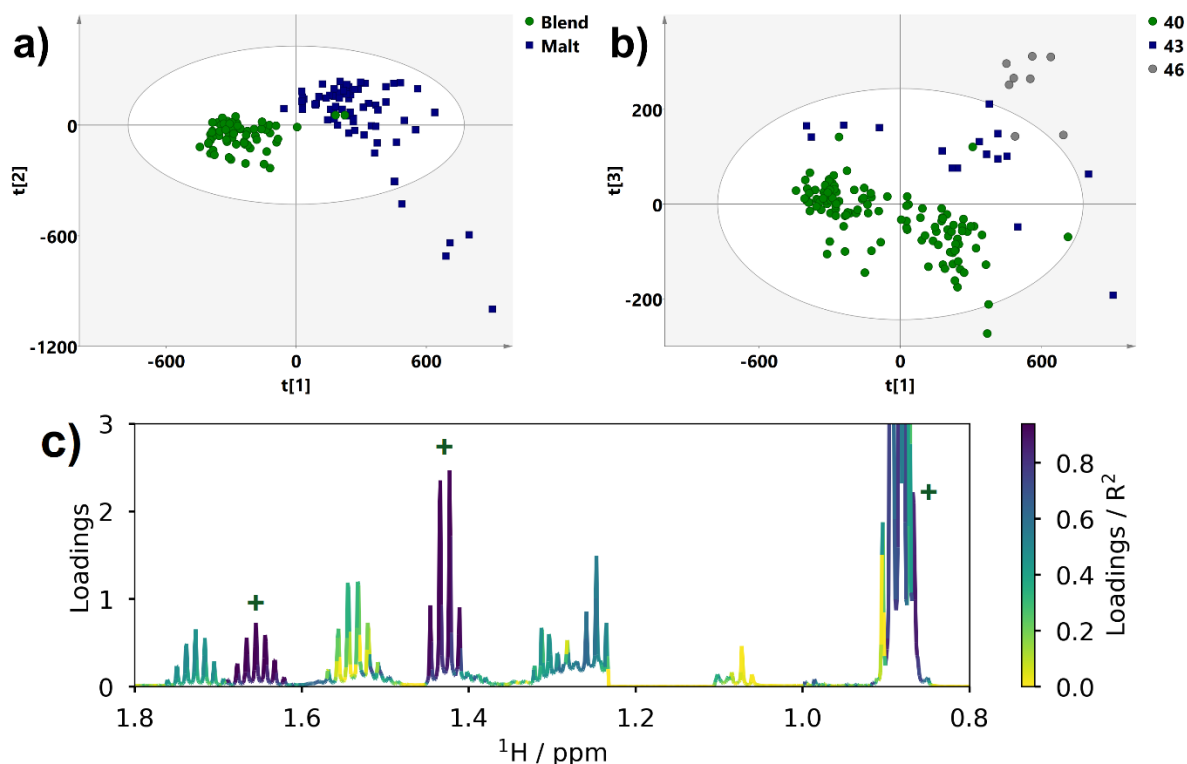


Figure 3 – PCA scores plots for a) components 1 and 2, colour coded according to blend or malt, and b) components 1 and 3, colour coded according to alcohol strength (ABV %). Ellipse represents Hotelling T^2 interval at 95%. PC1 represents 53% of variance, PC2 16%, and PC3 5%. The outliers in a) are samples matured in both Sherry and Bourbon casks exhibiting high levels of maturation related character. C) shows the s-line plot for OPLS-DA model classifying blend and malt scotch whiskies. The loadings and correlation between loadings and classification are shown between 1.8 and 0.8 ppm, indicating key signals discriminating blends and malt samples. The strongest signals correspond to 3-methylbutanol (annotated with a + symbol).

Figure 3a shows a near perfect separation of blended and malt whiskies, primarily through PC1. The blended whiskies are more tightly clustered, indicating less variation amongst this class than in the malt whiskies, where the samples are more disperse. Several of the malt whisky samples are outliers of the model and transpire to be whiskies matured in both Sherry and Bourbon casks (samples S10-1313, S12-2388, S13-0090, S14-1964, see Table S1), or finished in a Port pipe (sample S14-1972), and may have significantly more maturation-character than the rest of the sample set. Inspection of their spectra showed no obvious deficits in spectral quality, either through shimming or solvent suppression imperfections. A couple of the blended whiskies group with the malts; these samples were premium blends containing a high proportion of malt (relative to grain) spirit in the blend (S14-2858 and S13-

0110). The loadings for the first three principal components are shown in Figure S9-11. The loadings for PC1 (Figure S9) show a global positive trend indicating the samples on the positive side of PC1 (i.e., the malts) contain higher concentrations of most compounds observed by NMR. Malt whiskies are both produced with lower rectification during distillation and often premium products containing more maturation related compounds, and thus would be expected to contain more compounds as observed by NMR. The loadings for PC2 (Figure S10) show positive loadings for the higher alcohols including 2-phenylethanol, and negative loadings for the carbohydrates and some cask extractives such as furfural.

Interestingly, Figure 3b shows that PC3 can seemingly separate out, approximately, samples based on their alcohol strength. Inspection of the loadings for PC3 (Figure S11) indicates a few possible causes for this separation. The largest loadings are around 3.35 ppm and may correspond to signals from methanol, iso-butanol, and other unknown compounds, as well as at 0.88 ppm corresponding to the methyl signals from the higher alcohols. Furthermore, moderately large loadings are observed for ethyl acetate (4.13 ppm) and another unknown set of signals around 3.55 ppm. It is possible that the alcohol strength variation induces changes in the relative composition of the NMR detectable compounds – either directly or indirectly by affecting the equilibrium of compounds existing in multiple forms. It is also possible that the alcohol strength induced subtle changes in chemical shifts not accounted for by peak-alignment or binning of the data are behind this distinction. The effects of chemical shift variations on chemometric analysis have been recently investigated (Cañueto, Salek, Correig, & Cañellas, 2018) and may be worth future analysis of this sample set.

3.2.3 OPLS-DA models whisky class, peat status, and maturation wood type

As PCA is an unsupervised technique, and not strictly a classification method, a more rigorous interrogation of the data requires an alternative technique. OPLS-DA was chosen for its ability to separate out orthogonal data from the model, and here is used to model various parameters of Scotch Whisky production including: blend vs malt; peated or unpeated; maturation wood type; age;

329 geographical origin and authenticity. These categorical data are summarised in Table S1; only samples
330 where the relevant class was known were used to construct each model. The results for these OPLS-
331 DA models, and the PCA model, are summarised in

332 Table 2. Scores plots for OPLS-DA can be misleading, especially when presented without additional
333 statistical analysis. In this case, a combination of CV-ANOVA and permutation testing was used to
334 confirm if a model could accurately model the data against the classes defined.

335

Table 2 – Summary of PCA and OPLS-DA models including the number of components, number of observations, cumulative R^2X , R^2Y , and Q^2 values, and F -statistics and p -values as determined from CV-ANOVA. OPLS-DA models have $a+b$ components, where a is the number of predictive and b the number of orthogonal components. Wood models are explained in the text, with the number in brackets referring to the number of wood types modelled. Authenticity models are discussed in the following section, model 1 used the whole spectral width and model 2 use only the 6-10 ppm region.

Model	Component s	n	R^2X	R^2Y	Q^2	F	p
PCA	17	148	0.95		0.88		
PCA	3	148	0.74		0.70		
Blend/Malt	1+3	148	0.76	0.94	0.92	197	<0.05
Peat	1+4	148	0.72	0.85	0.74	38.7	<0.05
Wood (4)	3+6	60	0.87	0.81	0.54	2.3	<0.05
Wood (3)	2+2	57	0.71	0.59	0.36	3.3	<0.05
Wood (2)	1+1	57	0.52	0.50	0.39	8.4	<0.05
Age	3+0	69	0.49	0.26	0.10	1.2	0.244
Origin	2+4	69	0.78	0.48	0.25	1.2	0.263
Authenticity PCA	18	180	0.89		0.62		
Authenticity (1)	1+6	180	0.58	0.97	0.83	55.3	<0.05
Authenticity (2)	1+3	180	0.40	0.81	0.73	57.4	<0.05

The first classification was again for blend vs. malt whisky. The separation shown in the scores plot (Figure S12) is a substantial improvement upon the PCA model (Figure 3), with perfect separation through the predictive component and yielding similar results in the orthogonal component – that the blends are more tightly clustered, and malts more disperse. In this model also the two high-end blends that were misclassified by PCA (see Figure 3a) were correctly classified. With $p < 0.05$, this model is deemed statistically significant. An S-line plot (Figure 3c) constructed to visualise the cause for this

separation shows that 3-methylbutanol is key in discriminating between blend and malt whiskies. As this compound is known to be far more abundant in malt spirit than grain spirit, it stands to reason it would be key in discriminating blends and malts. Indeed, this has been reported before, using traditional analytical techniques such as GC-MS (R. Aylott & Mackenzie, 2010).

The next model investigated peated versus unpeated samples, initially using the whole spectrum but subsequently using just data from between 6 and 10 ppm, as here most differences were expected to be found. CV-ANOVA indicated this model was successful ($p < 0.05$), however interpreting the precise cause was more challenging. The scores plot and an S-line plot were constructed (Figure S13) and shows minor signals in the aromatic part of the spectrum contributing to the separation – these are likely some phenolic, peat-derived compounds, however their identity is unknown due to their low abundance. Reference spectra for the major known peat-derived phenols are shown in Figure S14 for comparison. These show large signal density at around 6.85 ppm, where the S-line loadings are the strongest, nevertheless, this comparison was inconclusive towards identification of the NMR markers for peat.

In our previous work using FTICR MS (Kew, Goodall, et al., 2017), discrimination of maturation wood types – Bourbon, Sherry, or a mix of the two – was successful. This was repeated here using the NMR data in three models: wood (4), wood (3), and wood (2). These correspond, respectively, to a four-class model – Bourbon (B), Sherry (S), Bourbon and Sherry (BS), and Ale (A) casks – and then a three-class model (excludes Ale), and finally a two-class model – Bourbon and Sherry, where the mixed wood types have been reclassified as just Sherry. This final reclassification is justified on the grounds of trying to identify if a sample has spent any time in Sherry casks, rather than strictly discriminating between Bourbon and Sherry maturation. This also allows for larger sample sets and increases statistical rigour.

The four-class wood model (Wood (4), data not shown) suggested that the ale cask samples were outliers and relatively similar. This is consistent with only three ale cask samples being included, and all being the same product from different years of production. Despite the difficulties for multi-class

OPLS-DA models, CV-ANOVA scored this model as significant ($p < 0.05$). Visual inspection of the data highlighted signals which were present only in the ale cask matured samples and not in any of the other 145 NMR spectra acquired. The 1D ^1H , 2D COSY, and 2D ^1H - ^{13}C HSQC regions of interest for these signals are shown in Figures S15 and S16. These signals appear to be carbohydrate anomeric protons at *ca.* 5.2 ppm. They are poorly resolved, and likely represent a mixture of oligomeric carbohydrates related to beer production.

The subsequent wood models were constructed by first excluding the ale casks and yielded poorer R^2 and Q^2 values than the four-class model. Figure S17a shows the scores plots for the three-class (Wood (3)) model, which includes B, S, and BS samples. There is some overlap between B and BS samples, to be expected given the common use of Bourbon wood. This model discriminates between B and BS broadly on component 1, and S and B/BS on component 2. To simplify the model, BS samples were relabelled S for the two-class (Wood (2)) model, scores shown in Figure S17b. Reclassifying the BS samples, rather than excluding them altogether, allows the model to retain a large sample set. However, the question is now more focused on Sherry related characteristics, as both classes will contain some Bourbon wood. Again, for both two- and three-class models, statistical significance remained high ($p < 0.05$) and permutation testing for the three-class model demonstrated the validity of the model (Figure S18).

Reducing the OPLS-DA model to two classes aids interpretation. Examination of the S-Line plot (Figure S17c), possible to generate only for 2-class models, indicated several moderately correlated signals relating to this class separation, especially those of the higher alcohols and carbohydrates including glucose. Furthermore, many of the minor unresolved signals between 6 and 10 ppm appear to be correlated with this class separation, with the noticeable exception of 2-phenylethanol. 2-Phenylethanol is a fermentation by-product rather than a maturation related congener, and it is reasonable for it not to be correlated. The other compounds in this region are primarily maturation related – including syringaldehyde and furfural – and it is to be expected that they would be more

abundant in Sherry cask matured whiskies. Sherry cask matured whiskies are often deliberately styled to have a more mature characteristic. Additionally, the moderate correlation between the carbohydrate region and Sherry maturation may come from the wood, or possibly from carry over of the sweeter Sherry wines compared to the dry (no, or very little, residual sugar) Bourbon barrels.

Overall, NMR analysis of maturation related properties was not as successful as our previous efforts with FTICR MS (Kew, Goodall, et al., 2017). The likely reason behind this is a limited sensitivity and resolution achievable by NMR. In FTICR MS, we observe thousands of resolved peaks of varying concentrations, masses and volatilities. In NMR, far fewer peaks are well resolved, and largely correspond to the most abundant congeners. The low-abundance compounds introduced or modified during maturation represent too subtle a change for NMR to register.

Finally, two further models were constructed around age statements and geographical origins. Neither of these parameters was successfully modelled, with poor R^2 , Q^2 and p values. It is not surprising that these models failed – neither age nor geographical origin (within Scotland) should be able to be modelled as the chemistry of the whisky does not depend on either of these directly. Age, for example, will increase the mature characteristics of a spirit, however, cask activity will also be significant – 20 years in a cask previously used for Scotch Whisky maturation (a refill cask) can yield a less mature spirit than 10 years in a cask which is fresh to the Scotch Whisky industry (a first fill cask) (Piggot et al., 1993). Likewise, geographic origin within Scotland does not dictate any process parameters – whilst Islay whiskies are stereotypically very peated, several distilleries produce non-peated malt whisky on Islay, and many distilleries outside of Islay also produce peated malt whisky. Scotch Whisky does not have *terroir* in the same way as wine – none of the raw materials required have to be sourced locally, and maturation often occurs in warehouses in other parts of Scotland – and thus generic geography is inherently difficult or impossible to model. That said, specific distilleries may be able to be modelled, provided they produce a consistent product.

3.3 NMR FOR GENERIC SCOTCH AUTHENTICATION

The acquired reference library of 148 authentic final product Scotch Whisky samples with excellent provenance (sourced directly by the producers) provided the opportunity to model genuine Scotch Whisky and to compare it to 32 available counterfeit samples. There are many possible approaches to address this problem, including compound concentration ratio analysis (R. Aylott & Mackenzie, 2010), statistical analysis, and machine learning approaches. Here, PCA and OPLS-DA analysis of ^1H NMR was used. Because of the sometimes-large differences in the NMR spectra of the real and fake samples, no post-processing peak alignment was used for this model.

First, a PCA model was constructed for all 180 spectra ($n_{\text{real}}=148$, $n_{\text{fake}}=32$) using the whole spectral width. The first two components described only 36% of the variance in the data, and 18 components were required to describe 89% of the variance, showing the data had large variation. This is due to the large differences between the real and fake samples, and within the set of fake samples. Still, the PCA scores plot (Figure S19) shows some separation between the real and fake samples. The real samples separate into two broad clusters, with the top left cluster containing samples finished in Sherry casks and having more sugar. The loadings for this model (not shown) separate out PC1 based on sugars and higher alcohols, whilst the PC2 separation is less easily understood.

To better classify samples, and understand the classification, OPLS-DA models were constructed. The first model is based on the analysis of whole spectra and successfully separates out real and fake samples (Figure 4a), with permutation testing (Figure S20) and CV-ANOVA ($p<0.05$) supporting the validity of the model. The S-Line plot (Figure 4c) confirms what was observed for the PCA loadings – that the major discriminating factors between the real and fake samples in this model were the presence of higher alcohols in real samples, and greater levels of carbohydrates in the fakes. Furthermore, the fakes contained NMR-detectable levels of glycerol, which was not observed in any of the authentic samples. Glycerol, and sugars, may be added to the fakes, and the levels observed in

the fakes would not normally be expected in the authentic samples (R. Aylott & Mackenzie, 2010; Kuballa et al., 2018).

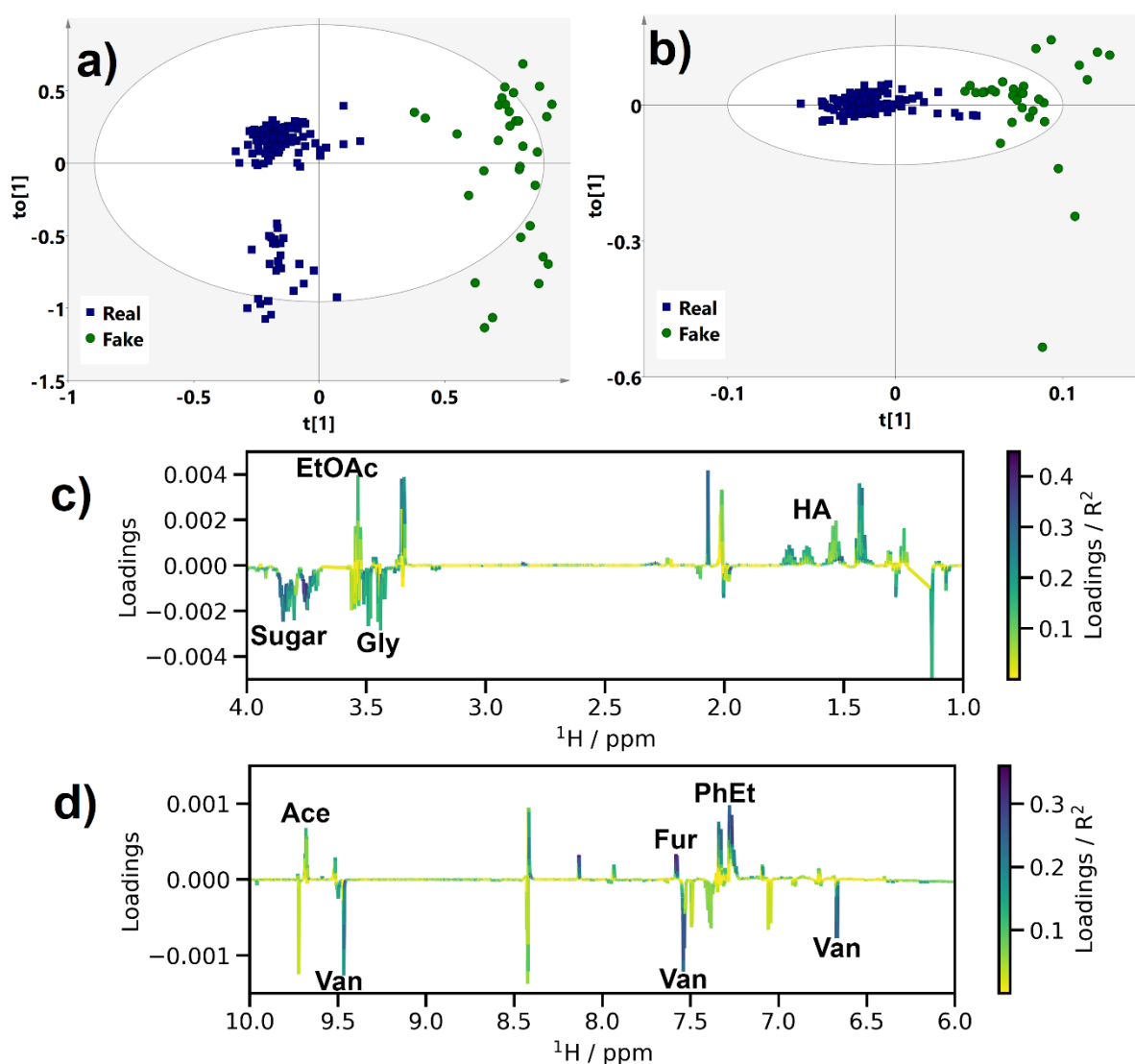


Figure 4 – OPLS-DA scores plots (a-b) for real and fake Scotch Whisky samples and S-line plots (c-d). The first model used the entire spectral width (with only selected region of interest shown) (a, c), while the second analysed only the 6-10 ppm region (b, d). Key compound signals are labelled – ‘sugar’ are sugars including glucose and fructose, ‘EtOAc’ is ethyl acetate, ‘Gly’ is glycerol, ‘HA’ are higher alcohols, ‘Ace’ is acetaldehyde, ‘Van’ is vanillin, ‘Fur’ is furfural, and ‘PhEt’ is 2-phenylethanol.

Finally, an OPLS-DA model was constructed from just the lower intensity signals between 6 and 10 ppm as this region is expected to contain more maturation related signals, as well as to determine if less abundant signals could be used for modelling. This model was also successful, with permutation testing (Figure S21) and CV-ANOVA ($p < 0.05$), again supporting the model. The scores plot (Figure 4b)

shows a major outlier at the bottom of y-axis (orthogonal component), which transpired to have a very high level of vanillin present. The S-Line plot for this model (Figure 4d) showed that the counterfeits could be discriminated based on this spectral region for both excessive levels of some compounds (e.g. vanillin) and insufficient levels, or a complete absence of, expected compounds such as 2-phenylethanol and furfural.

Overall, NMR spectroscopy proved to be a simple and accurate means to discriminate between counterfeit and authentic samples of Scotch Whisky. With larger sample sets, including more unusual authentic samples, this approach could be further validated, however as a proof of concept for generic authentication, these results are promising. The results here suggest that counterfeiters are adding chemicals to alcoholic spirits, to enhance the organoleptic properties of their base spirit. However, the chemicals added are either not present in authentic samples or added at incorrect concentrations in relation to other compounds. .

4 CONCLUSION

NMR spectroscopy provides untargeted, quantitative insights into the diverse chemistry of Scotch Whisky. Acquisition of the “solvent” suppressed 1D ^1H NMR spectra of Scotch Whisky is routine and fast, and the suite of complementary 1D and 2D NMR experiments allowed for the structural characterisation of dozens of compounds in Scotch Whisky.

Many compounds in Scotch Whisky were identifiable by comparison to reference and spike-in NMR spectra, supported by homo- and heterocorrelated NMR experiments when required. Some compounds, which exist in multiple forms in solution, were investigated in more detail. The characterisation of α and β epimers of glucopyranose, fructopyranose and fructofuranose was successful. Two hemiacetal forms of acetaldehyde were identified by means of several NMR experiments, including DOSY, whilst other, less significant, acetaldehyde equilibrium products were observed in the ^1H spectra but remain uncharacterised. Quantification of congener compounds in

Scotch Whisky was hindered by variable signal overlap and peak position, current software limitations, and for some compounds existence of several structural isomers.

Statistical analysis of NMR spectra of Scotch Whisky based on the analysis of 148 samples provided insight into chemical identification, origins, and sample discrimination. Binning of the spectra was performed uniformly with a fine bin width (0.001 ppm), allowing for retention of ^1H multiplicities whilst reducing the data matrix size considerably. STOCSY was utilised to correlate signals from the same compound or compounds with similar origins, whilst ICA decomposed the NMR spectra into compound spectra with some success. Classification analysis of Scotch Whiskies was performed with PCA and OPLS-DA in terms of different variable. Blend or malt status, and maturation wood type were successfully modelled, whilst age and geographical origin could not be modelled. The statistical models were interrogated for validity and chemical reasoning behind the outcomes of these models.

Finally, generic authenticity of Scotch Whisky was modelled successfully using OPLS-DA and a set of counterfeit samples. Using both the whole spectrum and just the 6-10 ppm region yielded successful results, and the main discrimination variables were the addition of flavour enhancing compounds to the fakes – including sugars, glycerol, and vanillin – at levels or ratios not found in authentic Scotch Whisky.

Raw and processed NMR data of the genuine Scotch Whisky samples will be available from DOI:XXX after a one-year embargo.

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6 CONFLICT OF INTEREST

WK was funded, in part, by SWRI; IG is employed by SWRI. SWRI is the Scotch Whisky industry's Research & Technology Organisation; it is funded by its membership of Scotch Whisky production companies. SWRI's remit is to ensure sustainability of the industry and its supply chain, improve process efficiency and help protect the category. It does this by carrying out a comprehensive programme of pre-competitive and applied research.

7 SUPPLEMENTARY DATA

Supplementary data, as discussed in the text, is available online at the publishers DOI:XXX

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